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# ELECTRIC FIELD EFFECTS ON IMMOBILIZED UREASE ACTIVITY

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## Summary

The behavior of an enzyme/membrane system containing urease is studied when an external electric field is applied. The device using a potential difference across the enzyme/membrane system is first described. Optimal operating conditions with respect to substrate concentration, ionic strength and pH are studied. Possible mechanisms of the change in membrane activity by electric field are discussed.

## Introduction

The increasing use of immobilized enzymes in recent years has lead to a large number of studies which have been concerned with their applications to clinical analysis and industrial processes [1—3].

The study of an ionic substrate diffusion inside an enzymically charged membrane allows to point out the kinetic disturbances due to the electrostatic field established by the charged support [4].

A theoretical analysis has been made concerning the interaction between an electromagnetic field and a planar localized enzyme system [5]. Nevertheless, the resulting mathematical model does not take into account the diffusion-reaction equations inside an active layer.

An attempt of the activity control of immobilized enzyme by means of an electric field has also been made. The enzyme is entrapped together with a liquid crystal in a collagen membrane [6,7]. These liquid crystal molecules arrange themselves regularly under an electric field. However, the mechanism of modification of the enzymic activity is far from being understood [6,7].

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Our aim is to study the behavior of an enzymic membrane obtained by the cross-linking of the enzyme together with an inert protein (albumin) when an electric field is applied.

The device using an applied potential difference across the enzymic membrane by means of two platinum electrodes is first described. Then the effects of substrate concentration, ionic strength and pH are discussed. An attempt to explain the phenomena is made by coupling the diffusion-reaction equations of substrate inside the active layer with the migration of ionic products of the reaction under the electric field.

## Materials and Methods

*Materials*

Urease (powder from jack beans, type IX, 5.1 Sigma units/mg) was obtained from Sigma. One Sigma unit is the amount of enzyme that will liberate 1 mg ammonia nitrogen from urea in 5 min at pH 7 at 30°C. Other reagents were commercially available analytical reagents or laboratory grade materials. Deionized water was used in all procedures.

*Preparation of urease-albumin membrane*

50 mg enzyme were dissolved in 2 ml 17.5% human albumin. Then 0.2 ml 25% glutaraldehyde was added and the solution stirred for 10 s. Part of this solution was deposited on a nylon grating (45 × 65 × 0.2 mm) and left for cross-linking at 25°C for 20 min. The thickness of the resulting membrane was 1 mm. Before use, it was rinsed for a few hours with a phosphate buffer solution (pH 7.0) to elute excess bifunctional reagent. The activity yield is approx. 75% after immobilization. The resulting membrane-specific activity is 0.4 Sigma unit per mg determined after grinding the membrane to fine pieces to eliminate diffusion phenomena.

*Determination of enzymic reaction rate*

The enzyme urease (urea amidohydrolase, EC 3.5.1.5) catalyzes the hydrolysis of urea to carbon dioxide and ammonia. Considering the acid-base properties of the reaction products, the reaction is:

$$O=C(NH_2)_2 + 2\,H_2O \rightarrow H_2CO_3 + 2\,NH_3$$

$$H_2CO_3 \overset{K_1}{\rightleftarrows} HCO_3^- + H^+ \qquad 2\,NH_3 \overset{K_3}{\rightleftarrows} 2\,NH_4^+ + 2\,H^+$$

$$HCO_3^- \overset{K_2}{\rightleftarrows} CO_3^{2-} + H^+$$

$$K_1 = 4.57 \cdot 10^{-7}$$

$$K_2 = 5.62 \cdot 10^{-11}$$

$$K_3 = 6.16 \cdot 10^{-10}$$

Measurements of the urea decomposition rate ($d[S]/dt$) were carried out using the pH-stat method (PHM 64 pH meter, Titrator TTT60, Autoburette ABU12, Recorder REC 61 servograph, all from Radiometer). To calculate the experimental reaction rate, the amount of neutralising acid added ($d[H^+]/dt$) has to be corrected to take into account the ionization of the products [8]:

$$\frac{d[S]}{dt} = \frac{d[H^+]}{dt} \times g([H^+])$$

where $g$ is a function of $K_1$, $K_2$ and $K_3$.

In all experiments, the volume of neutralising acid added is small compared with the volume of circulating solutions and can then be neglected.

Another correction has been made to take into account the urea consumption according to the first-order kinetics occurring with the substrate concentration used

$$\left(\frac{d[S]}{dt}\right)_{corrected} = \left(\frac{d[S]}{dt}\right)_{experimental} \times \frac{[urea]_{initial}}{[urea]_{initial} - [urea]_{consumed}}$$

*Procedure*

The cell is divided in four separate compartments A, B, C, D as shown in Fig. 1. The volume of each compartment is approx. 5 ml. The enzymic membrane separates both compartments A and B which contain the substrate solution. Every compartment (A, B, C and D) contains an ionic solution ($K_2SO_4$)

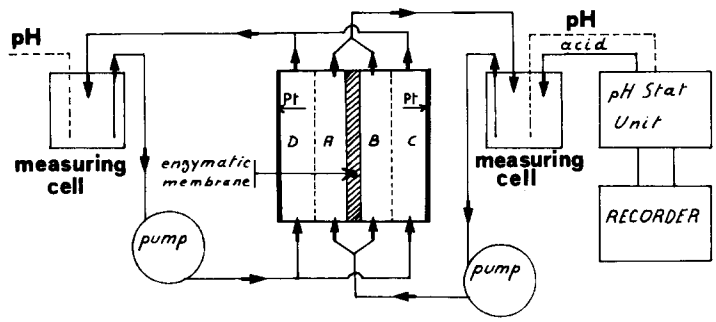


FIGURE 1a

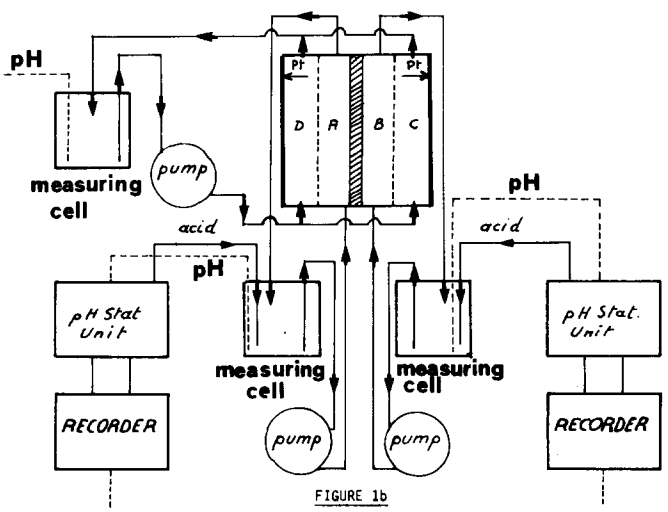


FIGURE 1b

Fig. 1. Determination of urease membrane activity under an electric field. Compartments A and B are connected (1a), compartments A and B are separated (1b).

which ensures the electric connection between the platinum electrodes. Two hydrophilic membranes, (Sartorius Membranfilter, Cellulose nitrate Ref. SM121-36) on either side of the enzymic layer, separate compartments A from D and B from C. This device prevents the electrochemical reaction of substrate on the electrodes. It eliminates the electrochemical production or consumption of $H^+$ from water which would interfere with the neutralising acid added by the pH-stat in the compartments A and B.

The cell is connected to a measuring unit (Fig. 1) which ensures: (i) a constant voltage across the platinum electrodes by means of a voltage stabilized power supply (Nobatron DCR 60-13 from Sorensen); (ii) the current intensity measurement; (iii) a thermostatic control of all the circulating solutions (25° C).

The enzymic reaction results in two fluxes of products on both sides of the enzymic membrane: $J_A$ towards compartment A and $J_B$ towards compartment B.

The study is carried out in two steps:

(1) The enzymic decomposition of urea is followed by measuring the sum of both fluxes $J_A$ and $J_B$. Compartments A and B are then connected (Fig. 1a), and only one pH-stat is used.

(2) The fluxes $J_A$ and $J_B$ are measured separately in each compartment A and B which are then not connected (Fig. 1b). Two pH-stat units are used. In all experiments, compartments C and D are connected.

The electrodes and the enzyme/membrane system have an effective surface of 9 $cm^2$. The liquid flow velocities (forced convection) are the same in every compartment and maintained large enough to allow sufficient supply of the substrate to the enzymic reaction (100 ml $\cdot$ $min^{-1}$).

During the measurements, the modification of the potential difference between the electrodes gives rise to an evolution of the reaction rate towards a new stable value corresponding to a new steady-state all over the active layer. This results in a new straight line on the recorder of the pH-stat unit.

## Results

The experimental device allows to mix solutions A and B (Fig. 1a) and solutions C and D (Figs. 1a and b) in order to prevent any change in ionic species concentrations due to electromigration from one compartment to another. Initially, every compartment (A, B, C and D) contains the same $K_2SO_4$ solution with a given pH and a given concentration. The substrate is introduced into the solution AB with a Gilmont ultra-microburet containing 0.1 M urea solution. No significal diffusion of urea from solution AB to solution CD has been observed during experiments.

Measurements of the current strength through the circuit as a function of applied potential difference ($\Delta V$) have been carried out for various $K_2SO_4$ concentrations ($3.45 \cdot 10^{-3}$, $1.72 \cdot 10^{-3}$, $5.75 \cdot 10^{-4}$, $4.31 \cdot 10^{-4}$ M). When the potential difference is lower than 2.2 V, the current intensity is nearly zero, whatever the ionic strength. For higher values, the intensity is proportional to $\Delta V$, for a given salt concentration. For a given $\Delta V$ value, the higher salt concentration, the higher the intensity.

The reaction rate as a function of applied potential difference, for various

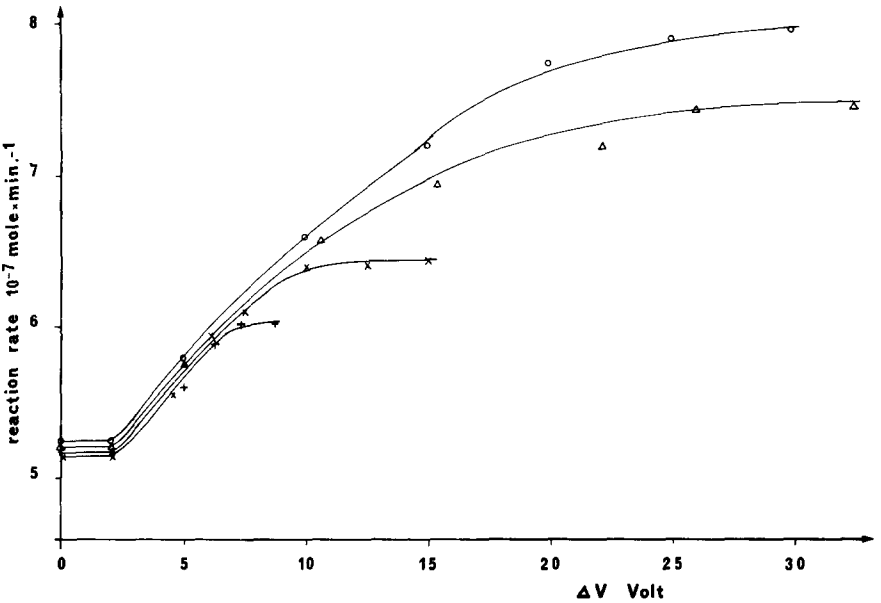


Fig. 2. Reaction rate as a function of applied potential difference ($\Delta V$) for various potassium sulfate concentrations. (+) $3.45 \cdot 10^{-3}$ M, (X) $1.72 \cdot 10^{-3}$ M, (△) $5.75 \cdot 10^{-4}$ M, (○) $4.31 \cdot 10^{-4}$ M. Measurements were done at pH 5.02 with a $10^{-3}$ M urea solution.

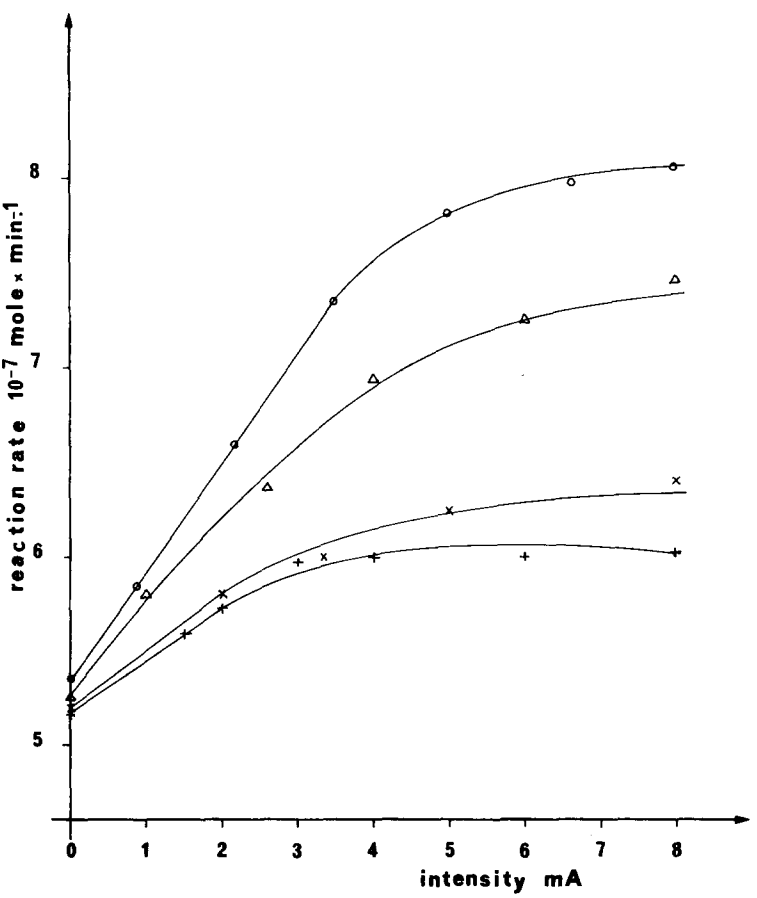


Fig. 3 Reaction rate as a function of current intensity for various potassium sulfate concentrations. (+) $3.45 \cdot 10^{-3}$ M, (X) $1.72 \cdot 10^{-3}$ M, (△) $5.75 \cdot 10^{-4}$ M, (○) $4.31 \cdot 10^{-4}$ M. Measurements were done at pH 5.02 in a $10^{-3}$ M urea solution.

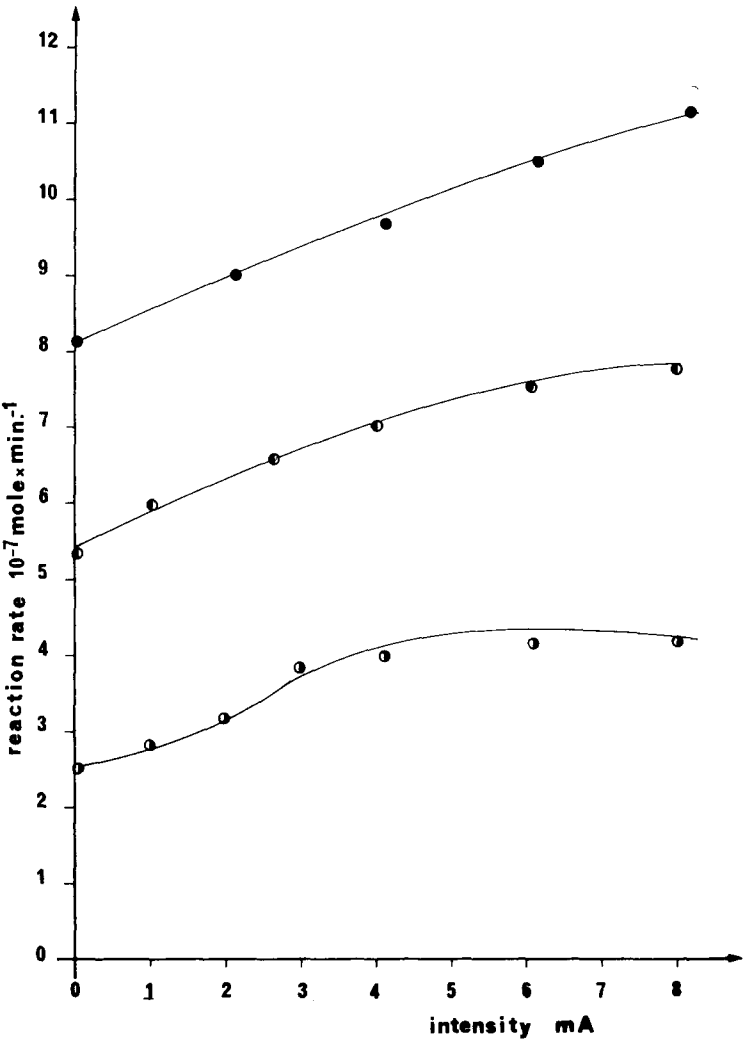


Fig. 4. Reaction rate as a function of current intensity for various urea concentrations. (◑) $5 \cdot 10^{-4}$ M, (◐) $10^{-3}$ M, (●) $1.5 \cdot 10^{-3}$ M. Measurements were done at pH 5.02 in a $5.75 \cdot 10^{-4}$ M potassium sulfate solution.

$K_2SO_4$ concentrations is shown in Fig. 2. The enzymic reaction rate is unchanged for an applied potential difference ranging from 0 to 2.2 V, corresponding to the absence of current in the circuit. For a given potassium sulfate concentration, the reaction rate is linear for low potential difference. For higher values, the reaction rate tends towards a plateau. The reaction rate is a function of $K_2SO_4$ concentration for a given potential difference (Fig. 2).

The reaction rate as a function of current intensity, for various $K_2SO_4$ concentrations is shown in Fig. 3. For a given current intensity, the reaction rate decreases when $K_2SO_4$ concentration increases. For a given salt concentration, the reaction rate is linear for low current intensity. For higher values, the reaction rate tends towards a plateau.

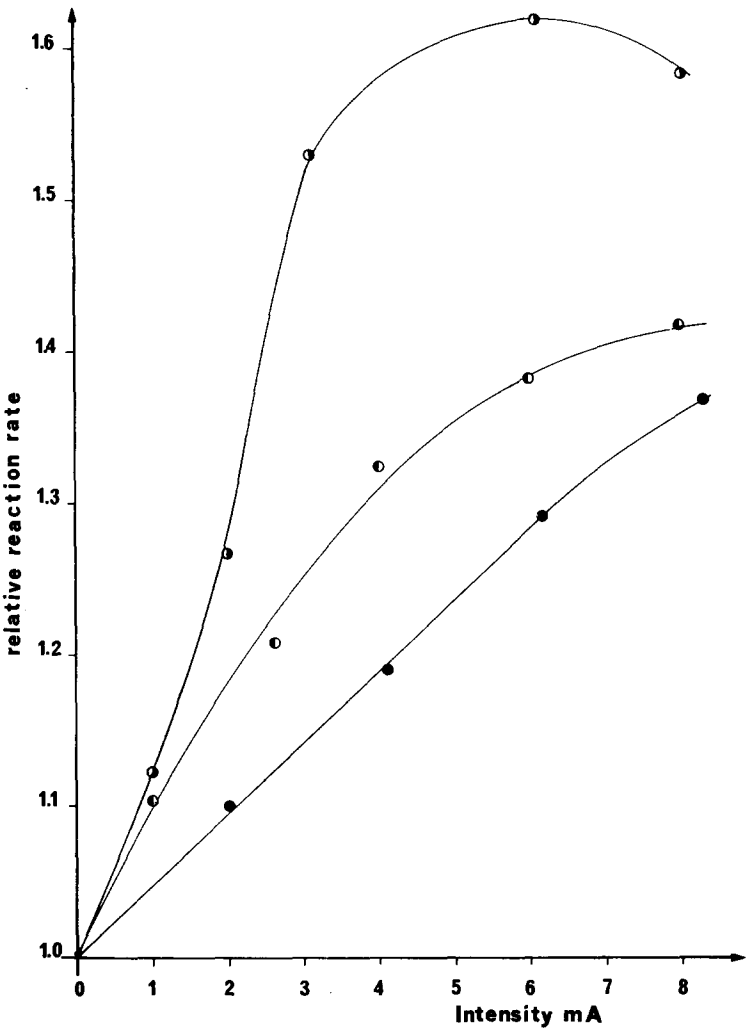


Fig. 5. Relative reaction rate as a function of current intensity for various urea concentrations. Concentrations and symbols as in Fig. 4.

The enzymic decomposition of urea gives rise to ionic species in solution AB, involving a change in its conductivity. This ion production must be slight compared with the $K_2SO_4$ concentration, in order to keep a constant current intensity for a given potential difference. However, as shown in Figs. 2 and 3, an increasing $K_2SO_4$ concentration decreases the effect of the current intensity on the reaction rate. The following studies have been performed with a $5.75 \cdot 10^{-4}$ M $K_2SO_4$ solution.

The reaction rate as a function of current intensity, for various substrate concentrations ($0.5 \cdot 10^{-3}$, $1 \cdot 10^{-3}$, $1.5 \cdot 10^{-3}$ M) is shown in Fig. 4. In the absence of the external electric field, the reaction is first-order with respect to the substrate. The reaction rate increases with an increasing current intensity.

If $\tau$ expresses the relative reaction rate ($\tau$ = reaction rate for a given intensity

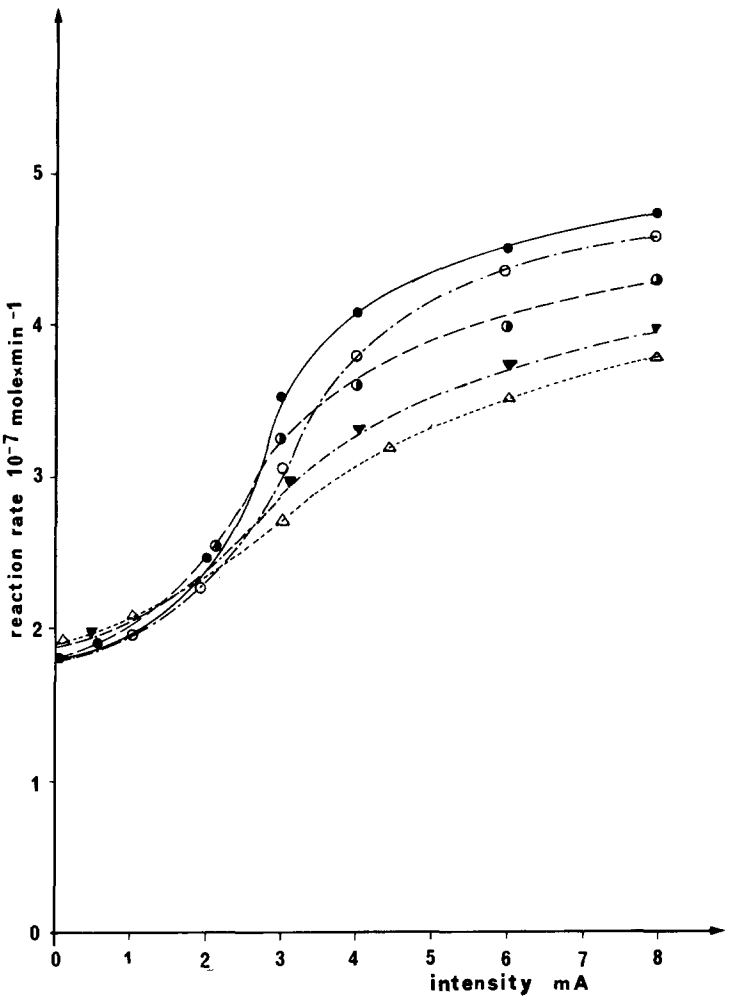


Fig. 6. Reaction rate as a function of current intensity for various pH values of the substrate solution. (○) 6.45, (●) 6.00, (◑) 5.40, (▼) 4.70, (△) 4.48. Measurements were done with $5.75 \cdot 10^{-4}$ M $K_2SO_4$ in a $5 \cdot 10^{-4}$ M urea solution.

$I$/reaction rate for $I = 0$), Fig. 5 shows the variation of $\tau$ as a function of current intensity for urea concentration = $0.5 \cdot 10^{-3}$ M, $1 \cdot 10^{-3}$ M, $1.5 \cdot 10^{-3}$ M. The increase in $\tau$ is larger when the urea concentration is $0.5 \cdot 10^{-3}$ M. In this case, the curve exhibits a maximum value ($\tau = 1.6, I = 5.5$ mA).

In order to favor the effect of the electric field on the enzymic reaction rate, next experiments are carried out with: [urea] = $0.5 \cdot 10^{-3}$ M and [$K_2SO_4$] = $5.75 \cdot 10^{-4}$ M.

The reaction rate as a function of current intensity for various pH values of the substrate solution is shown in Fig. 6. Before use, the pH values of the solutions containing $K_2SO_4$ and urea were adjusted with a 0.01 M $H_2SO_4$ solution. For pH values ranging from 3.6 to 6.45, the added $H_2SO_4$ can be neglected compared with the $K_2SO_4$ concentration.

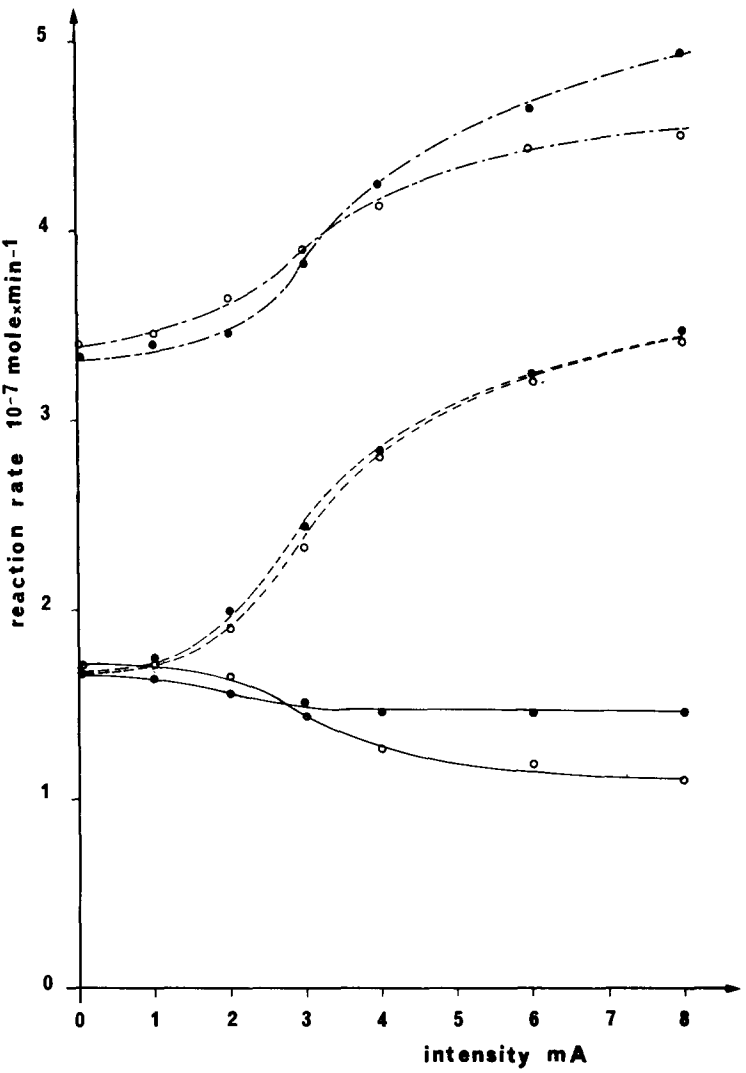


Fig. 7. Reaction rate in anodic (———), cathodic (- - - - -) compartments, and the corresponding sum of these two reaction rates (· — · — ·) for two pH values of the substrate solution (●) 5.80, (○) 4.49. Measurements were done with $5.75 \cdot 10^{-4}$ M $K_2SO_4$ in a $5 \cdot 10^{-4}$ M urea solution.

The reaction rate exhibits a sigmoid shape. For $I = 0$, the reaction rate is nearly $1.85 \cdot 10^{-7}$ mol · min$^{-1}$. When the current intensity is higher than 4 mA, the change in enzyme activity is maximal for pH 6.00. For other pH values, the increase in reaction rate is not so large.

Inside the active layer, the electric field results in an electromigration of anions towards the anode and cations towards the cathode. This phenomenon is then coupled with the ionic species production due to the enzymic reaction. Consequently, the change in products concentration is different in both compartments A and B. To point out the possible unsymmetrical production by the active membrane, the experimental device described in Fig. 1b is used. Both

compartments A and B are not connected, two pH-stat units are used. The surface of the membrane in front of the anode delimits the anodic compartment, the one in front of the cathode delimits the cathodic compartment.

The enzymic reaction rate in anodic, cathodic compartments and the corresponding calculated sum of these two reaction rates as a function of current intensity for two pH values (5.80—4.45) is shown in Fig. 7.

The reaction rates corresponding to the anodic compartment decrease slightly, when the current intensity increases.

The reaction rates corresponding to the cathodic compartment increase quickly between 2 and 4 mA and then tend towards a plateau.

The curves representing the sum of the two reaction rates corresponding to both compartments can be compared to those previously described (Fig. 6).

## Discussion

The modification of the enzymic reaction rate when an electric field is applied may result from:

(1) structural variation of the albumin-urease membrane, inducing a change in diffusion coefficients of substrate and products.

(2) conformational change in enzyme structure.

(3) pH variation inside the active-layer inducing a change in $V_m$ and $K_m$ [8].

One of these phenomena may have a predominant feature according to the range of electric field applied.

In the case where the enzyme irreversibly transforms one substrate into two products P and Q, the reaction can be written:

$$E + S \underset{k-1}{\overset{k+1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P + 2Q$$

The reaction rate can be written:

$$v = -\frac{\partial [S]}{\partial t} = \frac{\partial [P]}{\partial t} = \frac{\partial [Q]}{2\partial t} = V_m \frac{[S]}{K_m + [S]}$$

where $V_m$ is the maximum reaction rate and $K_m$ the Michaelis constant.

Inside the active-layer, concentrations of substrate and products are controlled by diffusion coupled with the reaction. This effect can be illustrated by the following equations:

$$\frac{\partial [S]}{\partial t} = D_S \frac{\partial^2 [S]}{\partial x^2} - V_m \frac{[S]}{K_m + [S]} \tag{1}$$

$$\frac{\partial [P]}{\partial t} = D_P \frac{\partial^2 [P]}{\partial x^2} + V_m \frac{[S]}{K_m + [S]} \tag{2}$$

$$\frac{\partial [Q]}{\partial t} = D_Q \frac{\partial^2 [Q]}{\partial x^2} + 2V_m \frac{[S]}{K_m + [S]} \tag{3}$$

where $t$ is the time of the reaction, $D_S$, $D_P$ and $D_Q$ the effective diffusion coefficients of S, P and Q inside the active layer which are assumed to be nearly equal for simplification and $x$ the distance of each point of the membrane to the external surface of this membrane.

The enzymic reaction results in a change in hydrogen ion concentration. Consequently, the pH inside the active layer will differ from that outside the membrane, owing to diffusional limitations on the egress of the products.

In the case where the enzyme is urease, we can write:

$$[P] = [CO_2]_t = [CO_2] + [H_2CO_3] + [HCO_3^-] + [CO_3^{2-}]$$

$$[Q] = [NH_3]_t = [NH_3] + [NH_4^+]$$

$$[Q] = 2[P]$$

where $[CO_2]_t$ and $[NH_3]_t$ are the total equivalent amount of $CO_2$ and $NH_3$ produced by enzymic reaction.

Inside the active layer, the pH value at any point depends on reaction product concentrations $[P]_x$ and $[Q]_x$. Considering the unbuffered enzyme system, it is then possible to calculate this pH value $pH_{(x)}$ from $[P]_x$ and $[Q]_x$. The enzymic reaction rate depends upon pH, mainly due to the polyelectrolytic properties of amino acids. In order to account for the pH dependency of $V_m$ and $K_m$, we substitute in Eqns. 1, 2 and 3 the following equations.

$$V_m\,(pH) = V_m^0\, f\,(pH)$$

$$K_m\,(pH) = K_m^0\, f'\,(pH)$$

where $V_m^0$ and $K_m^0$ are the enzyme parameters corresponding to the optimum pH (7.0 for urease), $f$ and $f'$ express the pH dependency of $V_m$ and $K_m$.

The local reaction rate inside the membrane is then determined by the local substrate and hydrogen ion concentrations. Considering the basic properties of reaction products, the pH inside the active layer is higher than that outside. The local reaction rate may then be smaller than that obtained with an optimal pH.

In the case where an electric field ($\vec{E}$) is applied, the concentration of S and P inside the active layer are controlled by diffusion coupled with reaction and with the electromigration of ionic species.

The flux of an ionic species $i$ can be written:

$$J_i = +\mu_i C_i \vec{E} - D_i \frac{\partial C_i}{\partial x} \tag{4}$$

where $\mu_i$ is the electric mobility of the ionic species $i$ and $D_i$ its effective diffusion coefficient, inside the membrane.

The evolution of its concentration is given by:

$$\frac{\partial C_i}{\partial t} = -\frac{\partial J_i}{\partial x} + V_m^0 \alpha \frac{[S]}{K_m^0 \beta + [S]} \tag{5}$$

where $\alpha$ and $\beta$ are coefficients depending upon pH and/or electric field values.

From Eqns. 4 and 5 we can write:

$$\frac{\partial C_i}{\partial t} = -\mu_i \vec{E} \frac{\partial C_i}{\partial x} - \mu_i C_i \frac{\partial \vec{E}}{\partial x} + D_i \frac{\partial^2 C_i}{\partial x^2} + V_m^0 \alpha \frac{[S]}{K_m^0 \beta + [S]} \tag{6}$$

As the electric current is transported by the ionic species, we can write:

$$d = \frac{I}{S'} = F \Sigma Z_i J_i \tag{7}$$

where $I$ is the current intensity, $S'$ the electrode area, F the Faraday and $Z_i$ the valence of the ionic species $i$.

The electric field can be calculated from Eqns. 4 and 7:

$$\vec{E} = \frac{d/F + \Sigma Z_i D_i \frac{\partial C_i}{\partial x}}{\Sigma Z_i \mu_i C_i} \quad (8)$$

The electric current results from migration of anions towards the anode, and cations towards the cathode. The total current is transported at once by $K_2SO_4$ and by the products of the enzymic reaction ($H^+$, $OH^-$, $HCO_3^-$, $CO_3^{2-}$, $NH_4^+$). When the $K_2SO_4$ concentration increases, the ratio of the current transported by the reaction products to that transported by $K_2SO_4$ decreases. This assumption agrees with the decrease in reaction rate for a given current intensity when the $K_2SO_4$ concentration increases (Fig. 3).

Inside the active layer, the electric field depends upon the ionic species concentration: the higher their concentration, the lower the resulting electric field (Eqn. 8). As the total amount of ionic products inside the active layer depends on substrate concentration according to a first order kinetic, low substrate concentrations increase electric field strength. This assumption agrees with the experimental results given by Figs. 5 and 6.

The applied electric field may result in a change in pH profile inside the membrane, and consequently in enzyme reaction rate.

From this point of view, the apparent reaction rate is higher on the cathodic side of the membrane than that on the anodic side (Fig. 7). This may be at once due to the unsymmetrical pH profile in the active layer and to the transport of ionic products through the membrane.

Numerical analysis on computer would allow to determine the profiles of pH and reaction products inside the membrane when an electric field is applied. The obtained results may result in a better understanding of observed phenomena.

## References


1 Wiseman, A. (1975) in Handbook of Enzyme Biotechnology (Wiseman, A., ed.), pp. 243—272, Ellis Howood, London
2 Barker, S.A. and Kay, I. (1975) in Handbook of Enzyme Biotechnology (Wiseman, A., ed.), pp. 101—110, Ellis Howood, London
3 Guilbault, G.G. (1976) Handbook of Enzymatic Methods of Analysis, Vol. 4, Marcel Dekker, New York
4 Goldstein, L., Levin, Y. and Katchalski, E. (1964) Biochemistry 3, 1913—1921
5 Seto, Y.S. and Hsieh, S.T. (1976) Biotechnol. Bioeng. 18, 837
6 Kubo, M., Karube, I. and Suzuki, S. (1976) Biochem. Biophys. Res. Commun. 69, 3, 731—736
7 Karube, I., Yugeta, Y. and Suzuki, S. (1977) Biotechnol. Bioeng. 19, 1493—1501
8 Atkinson, B., Rott, J. and Rousseau, I. (1977) Biotechnol. Bioeng. 19, 1037—1063